

UNITED STATES PATENT AND TRADEMARK OFFICE

IN THE APPLICATION OF:

McSwiggen et al.

Serial No. 10/665,951

Filed: September 18, 2003

**Title RNA Interference Mediated
Inhibition of Vascular
Endothelial Growth Factor and
Vascular Endothelial Growth
Factor Receptor Gene
Expression Using Short
Interfering Nucleic Acid (siNA)**

Examiner:

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Group Art Unit:

1635

Confirmation No.:

8325

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
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Sir:

1. I, James McSwiggen, am a named inventor of U.S. Patent Application Serial Number 10/665,951 and was formerly a Senior Research Fellow for Sirna Therapeutics, Inc., (“Sirna”) located at 2950 Wilderness Place, Boulder Colorado, 80301, the sole Assignee of USSN 10/665,951. I earned a B.S. with honors in Molecular Biology from the University of Wisconsin, Madison, in 1979 and a Ph.D. in Biochemistry and Biology from the University of Oregon, Eugene, in 1985. I have performed research in the field of siRNA and other nucleic acid technologies for over 25 years. A copy of my Curriculum Vitae is attached.

2. I am a named inventor on USSN 10/665,951, entitled “RNA Interference Mediated Inhibition of Vascular Endothelial Growth Factor and Vascular Endothelial Growth Factor Receptor Gene Expression Using Short Interfering Nucleic Acid (siNA)”.

3. I was employed as a research scientist at Sirna Therapeutics Inc. (formerly Ribozyme Pharmaceuticals Inc.) for 13 years. From 2000–2005, my work at Sirna involved, among other things, the analysis of gene sequences as possible siRNA targets, and the design of siRNA drugs

to attack those gene targets. From 1992–2000, my work as a Senior Scientist and Group Leader of the Enzymology/Biochemistry Department at Sirna involved the design and detailed analysis of chemically modified ribozymes. Specifically, my laboratory developed and applied novel modification strategies for ribozyme and antisense nucleic acid molecules for use in both *in vitro* and *in vivo* applications, including the use of such technologies in target validation and potential therapeutic applications. I have authored numerous publications and have been an inventor on several patents and patent applications involving the chemical modification of ribozyme and antisense nucleic acids (see attached Curriculum Vitae).

4. I was closely involved in the design strategies at Sirna to develop chemical modifications for increasing the nuclease stability of siRNAs while maintaining RNAi activity. The development of those strategies was not trivial, and there was nothing in the literature at that time to suggest a direction to take in order to achieve success, nor whether substantial modification of the siRNAs could even be successfully achieved. We received no guidance from the scientific literature that was published in 2000 to 2001. In fact, I believe that the literature of the day, and the general scientific community, tended to lead a person skilled in the art **away** from discovering such chemical modifications. I make this assertion, in particular, with respect to the work of Zamore et al., 2000 *Cell* 101(1):25-33, Bass, 2000 *Cell*, 101, 235–238, Elbashir et al., 2001 *Nature* 411(6836):494-8 (Elbashir 1) and Elbashir et al., 2001, *EMBO Journal*, 20:6877-6888 (Elbashir 2). These papers provided early guidance on the generic design of siRNAs for gene targeting, including analyses of requirements for length, structure, chemical composition, and sequence in order to mediate RNAi. One additional paper, Parrish et al. (*Molecular Cell*, 2000 Vol. 8:1077-1087), provided early insights into the RNAi phenomenon, but did not provide guidance in the areas of siRNA design; I will address this issue first.

5. **Parrish teaches nothing for siRNA design.** The paper by Parrish represents a broad survey of the biochemical properties of the RNAi reaction in nematodes using long dsRNAs, but it does not provide any useful information regarding the design of modified siRNA molecules. In 2000–2001 it was clear that RNAi was a conserved cellular mechanism that was present in a diverse set of organisms; it was first discovered in plants, then in nematodes, ciliates, fungi, *Drosophila*, and finally in mammalian cells (see for example Elbashir 1). But while the basic mechanism is conserved, it was clear to those skilled in the art that the mechanistic details could

be very different from one organism to another. Specifically, the lower Eukaryotes are easily activated by long dsRNA, while publications such as Elbashir 1 noted that long dsRNA failed to stimulate RNAi in mammalian cells; this was likely due to the activation of an interferon response in mammalian cells, which is absent in the lower Eukaryotes. Likewise, Bernstein et al (2001, *RNA* 7:1509-1521) noted that *C. elegans* and plants have a number of RNAi-related behaviors that are not found in mammalian cells, including the ability to pass the RNAi effect from one cell to the next, the ability to amplify the RNAi response such that a few dsRNA molecules can elicit a potent RNAi response, and the ability to pass the RNAi response from one cell generation to the next due to the long-lived nature of RNAi in these organisms (p1515-1516). These profound differences would teach those skilled in the art that it is unwise to generalize discoveries made in *C. elegans* to the world of mammalian RNAi.

6. A second factor that makes it difficult to draw lessons from Parrish is that all of the studies were performed using long dsRNA. The shortest dsRNA molecules used were 26 & 27 bp, but these were only used for initial base composition studies. In fact, Parrish clearly states that any molecules less than 26 bps were inactive (p1079, right column). The nucleotide modification studies were performed primarily using a 742 bp *unc-22A* sequence that apparently also contained “3–30 nt of dsRNA derived from polylinker sequences on each end, and polylinker-derived single stranded tails of 10–30 nt.” (Materials & Methods, p1085). The authors checked the annealing of these sequences by agarose gel, but that would only confirm that they were stuck together, not whether they were annealed properly. These long sequences add a great deal of ambiguity to the interpretation of the results. An “inactive” modification could be such because it failed to allow the strands to anneal properly rather than being deleterious to the RNAi machinery, and an “active” modification could actually be an inactive modification that is distributed sparsely enough on the sequence that the RNAi machinery can still function. This latter possibility is of particular concern since Parrish reports that they “were able to demonstrate interference activity following incorporation of any single modified residue”, but that “RNAs with two modified bases also had substantial decreases in effectiveness as RNAi triggers.” (p1081, right column). Thus, the modifications have a cumulative effect such as would be expected if the RNAi machinery was finding unmodified places on the long dsRNA to bind and activate.

7. One final argument against Parrish is that they themselves were unable to formulate a cogent conclusion to their chemical modification studies. They tested over 30 combinations of chemical modifications (their Figure 5, Figure 6, and data not shown), but in the discussion section they can only muster three short paragraphs speculating on the possible implications of these studies (p1084, left column). Their conclusions are: (1) the dsRNA might need to maintain an A-form helix to be active, (2) the antisense strand is more sensitive to modification than the sense strand, and (3) some modifications affect RNAi activity when added to either strand. These speculations are only weakly supported by the data. Coupled with the concerns mentioned above regarding long dsRNA and the difficulty of extending observations from *C. elegans* to mammalian cells, these considerations made it very difficult for us to draw any conclusions whatsoever from Parrish regarding the design of short siRNA molecules.

8. Returning, now, to the papers of Zamore, Bass, Elbashir 1 and Elbashir 2, I propose that these papers—and the general scientific community—tended to lead a person skilled in the art **away** from discovering the chemical modifications discovered by us in the current application. I believe there are three factors that tended to lead away from such a discovery. First, there was no motivation to seek such modifications. Second, these key papers from 2001 suggested that deviations from RNA in the siRNA duplex would lead to inactivation of the siRNA. Third, nothing in the literature gave guidance as to **how** to modify siRNAs, even if the motivation to seek such modifications was present and the expectation of failure was overcome. I will address these three points in order.

9. **No motivation to seek modifications.** Even from the early papers mentioned above, it was clear that short RNA duplexes could be potent initiators of RNAi in extracts and in cell culture. Elbashir 2 used 100 nM RNA duplexes in their experiments to achieve >90% knockdown in extracts, while later studies would observe efficient knockdown at 5-10 nM siRNA. siRNAs tested at Sirna and elsewhere typically have been 10-100 fold more potent than the majority of ribozymes or antisense molecules tested. With ribozymes and antisense it was clear that RNA stability would be critical to achieving optimal activity, even in cell culture. In contrast, the high potency of siRNAs tended to teach that no additional modification would be necessary, at least in cell culture. Also, it is common knowledge to those skilled in the art that single stranded RNA and DNA is much more susceptible to nuclease attack than double stranded

nucleic acids. Thus the relatively unstructured antisense and ribozymes would be expected to require additional stabilization, while the substantially double-stranded siRNA would not. An example of this thinking is seen in Elbashir 2, where an emphasis was placed on modifying the 3' single stranded ends of the siRNA, with little effort made to modify the double stranded 5' ends.

10. The methods paper of Elbashir 3 (Elbashir et al., 2002 Methods 26:199-213) best exemplifies the mindset of the day that additional chemical modifications are unnecessary for effective RNAi activity. This paper gives specific instructions for designing and carrying out an RNAi experiment. On page 202, Protocol 1 (step 2) states that:

Independent of the selection procedure described in Fig. 2, synthesize the sense siRNA as 5'-(N19)TT, and the sequence of the antisense siRNA as 5'-(N'19)TT, where N'19 denotes the reverse complement sequence of N19. N19 and N'19 indicate ribonucleotides; T indicates 2'-deoxythymidine.

Thus, RNA duplexes with dTdT 3' ends were considered the correct substrate for carrying out RNAi experiments. The terminal TT was there primarily to make chemical synthesis easier and less expensive, although some minor protection from **single-stranded** ribonucleases was also considered a possibility (Elbashir 2, Elbashir 3). Finally, Elbashir 3 makes specific mention of four suppliers of siRNA duplexes for RNAi research; all four companies supply the reagents in the standard form described in Protocol 1 of Elbashir 3.

11. **No expectation of success in chemical modification.** As stated above, there was no motivation to seek chemically modified siRNAs during the period in question, so it comes as no surprise that only a few papers discuss the subject. Elbashir 2 is the only paper from the period that describes a significant attempt to modify siRNAs away from their own standard of RNA with TT overhanging ends. Their efforts are incomplete, but also suggest that substantial modification will destroy RNAi activity. Under the heading "*The siRNA user guide*" (see page 6885) Elbashir 2 provides guidance to those of ordinary skill in the art on the design of siRNA duplexes. This guide states:

Efficiently silencing siRNA duplexes are composed of 21 nt sense and 21 nt antisense siRNAs and **must** be selected to form a 19 bp double helix with 2 nt 3'-overhanging ends. 2'-deoxy substitutions of the 2 nt 2'-overhanging ribonucleotides do not affect RNAi, but help to reduce the costs of RNA synthesis and may enhance RNase resistance of siRNA duplexes. **More extensive 2'-deoxy or 2'-O-methyl modifications reduce**

the ability of siRNAs to mediate RNAi, probably by interfering with protein association for siRNAp assembly

(emphasis added). This reference suggests that chemical modifications are generally not tolerated by siRNAs except for substitution of the 3'-terminal nucleotides of siRNA with deoxynucleotides. Further, modifications with 2'-O-methyl or other modifications were not tolerated.

12. Additionally, Elbashir 2 showed that modifications beyond the 3'-terminal nucleotides of the siRNA were not tolerated and provided further teachings that would have discouraged a person skilled in the art from introducing any chemical modifications to the nucleotides in the internal base-paired region of the siRNA duplex for the reasons set forth below (page 6886, right column, Elbashir 2):

Interestingly, substitution by 2'-O-methylribose, which adopts the ribose sugar pucker, also abolished RNAi, probably because methylation of the 2'-hydroxyls blocked hydrogen bond formation or introduced steric hindrance.

And on page 6885, right column, Elbashir teaches that RNAi activity is particularly sensitive to the nucleotides in the center of the siRNA:

Nucleotides in the center of the siRNA, located opposite to the target RNA cleavage site, are important specificity determinants and even single nucleotide changes reduce RNAi to undetectable levels.

and page 6884, right column,

The sequence changes located between the 3' end and the middle of the antisense siRNA completely abolished target RNA recognition, but mutations near the 5' end of the antisense siRNA exhibited a small degree of silencing.

These teachings regarding the sensitivity of RNAi activity to the nucleotides in the center of the siRNA, coupled with the reported effects of 2'-O-methylation, would have dissuaded those skilled in the art from introducing 2'-O-Me or other modifications into the center of an siRNA and/or would have prevented one skilled in the art from having a reasonable expectation of successfully obtaining an active siRNA with such a modifications.

13. Further, in the section entitled "2'-deoxy- and 2'-O-methyl-modified siRNA duplexes" (see pages 6881-6882), Elbashir 2 describes the effect of chemical modification on the activity of the siRNA duplex to mediate RNAi. The authors state,

To assess the importance of the siRNA ribose residues for RNAi, duplexes with 21 nt siRNAs and 2 nt 3'-overhangs with 2'-deoxy- or 2'-O-methyl-modified strands were

examined (Figure 4). Substitution of the 2 nt 3'-overhangs by 2'-deoxynucleotides had no effect and even the replacement of two additional ribonucleotides by 2'-deoxynucleotides adjacent to the overhangs in the paired region produced significantly active siRNAs. Thus, 8 out of 42 nt of a siRNA duplex were replaced by DNA residues without loss of activity.

Based on this work, we concluded that the use of 2'-O-methyl substitutions in siRNAs was not tolerated to maintain RNAi activity. While 2'-deoxy substitutions at the 3'-terminal positions were permitted, there was no mention of any active siRNAs using 2'-O-methyl modifications, even at the terminal positions. Furthermore, because complete substitution of one or both siRNA strands with either 2'-deoxy or 2'-O-methyl residues resulted in a complete loss of RNAi activity, we understood the results of Elbashir 2 to mean that modification of internal nucleotide positions reduced the ability of siRNAs to mediate RNAi, probably by interfering with protein interactions or siRNP assembly, blocking hydrogen bond formation or introducing steric hindrance (Elbashir 2, Pages 6885 and 6886).

14. The foregoing teachings therefore discouraged us and likely steered others away from exploring chemical modification of siRNAs beyond replacing the 3'-terminal positions with deoxynucleotides. When we further surveyed the literature, there was ample evidence to suggest that people in the art were using siRNA duplexes that were either unmodified or modified only at the two overhanging nucleotide positions at the 3'-end of the siRNA. The published reports available during the 2000-2001 time period also showed that all of the synthetic siRNAs being used were 21 nt siRNA duplexes with 19 base pairs and 3'-terminal 2'-deoxy substitutions, just as described in "*The siRNA users guide*" from Elbashir 2 (see for example Bitko *et al.*, 2001, BMC Microbiology, 1, 34 page 9, left column under heading Materials and Methods section; Kumar *et al.*, 2002, Malaria Journal, 1:5, page 9, right column, under heading Transfection by Inhibitory dsRNA; Holen *et al.*, 2002, Nucleic Acids Research, 30, 1757-1766, Figures 1, 2 and 6).

15. It was not until 2003 that scientists began to evaluate and report the use of chemical modifications other than 3'-terminal 2'-deoxy substitutions in siRNAs. See, for example, Chiu and Rana, 2003, RNA, 9:1034-1048 and Allerson *et al.*, 2005, *J. Med. Chem.* 48, 901. It is readily apparent from the publication record that those working in the RNAi field initially followed the teachings of Elbashir and others, outlined above in paragraph 10, in designing

siRNAs for experimental work. Only more recently has the use of more extensive chemical modifications become generally accepted.

16. We therefore carried out our initial experiments (during about 2000–2001) using the siRNAs used in the art by others, such as those set forth in Zamore, Elbashir 1, Elbashir 2 and others. We soon recognized, however, that these siRNAs had limited utility for more extensive applications in silencing of target genes in whole organisms, as such siRNAs were rapidly degraded by nucleases and possessed unfavorable pharmacokinetic and pharmacodynamic activity.

17. **No guidance on how to modify siRNAs.** It is incorrect to suggest that the teachings on the chemical modification of ribozymes and antisense would provide a roadmap for the ordinary person skilled in the art to be able to design stabilized siRNAs without loss of RNAi activity. Our own experience at Sirna argues very much to the contrary. With respect to our research, we found that chemical modification strategies for ribozymes differed greatly from those useful in antisense. For example, the modification strategy for ribozymes is dependant upon allowing the ribozymes to maintain catalytic activity, *e.g.*, by selective modification of the ribozyme binding arms and catalytic core. It required several years of research to arrive at modified ribozymes that were not only nuclease resistant but also maintained their catalytic activity (see for example Beigelman *et al.*, 1995 *The Journal of Biological Chemistry* 270:25702-25708). On the other hand, the modification strategy for antisense is dependent upon maintaining their ability to activate RNase H, and considerable research was involved in arriving at modified antisense molecules that were both nuclease resistant and maintained the ability to activate RNase H (see for example Monia *et al.*, 1993 *J. Biol. Chem.* 268:14514-14522).

18. The following examples illustrate some of the many differences between ribozymes, antisense and siRNA. (1) Both ribozymes and antisense are substantially single-stranded prior to interacting with their target, while siRNA is almost completely in a duplex form; it is well known to those skilled in the art that single-stranded nucleic acid is more susceptible to nuclease attack than is double-stranded nucleic acid. (2) Ribozymes and antisense will tolerate substantial 5' and 3' terminal modifications, an observation that we have used to good effect to protect these molecules from exonuclease attack (*c.f.* Beigelman *et al.*); in contrast, the activity of siRNAs are almost completely destroyed by blocking the 5' end of the antisense strand of the siRNA with

chemical modification. (3) The activity of an antisense molecule is destroyed by modifications that alter the DNA-like structure at the core of molecule; in contrast ribozymes form a complex RNA secondary structure to be active. It was not clear in 2001 whether the siRNA duplex would need to maintain an RNA-like structure or whether other structures would be permitted. (4) Antisense molecules and ribozymes are active in the nucleus, while the RNAi activity occurs in the cytoplasm.

19. Because of our experience with modified ribozymes and antisense oligonucleotides, and understanding that the mechanism of RNA interference was different than both of these technologies, we had no faith that the results we observed with modifications of ribozymes and antisense oligonucleotides would inform us regarding the effects of chemical modifications in RNA interference. Accordingly, when we began investigating RNA interference technologies for targeted gene silencing we started from scratch, evaluating systematically the position and type of chemical modifications that siRNAs could tolerate without significantly diminishing the ability to mediate RNAi. In addition, the foregoing references (e.g., Elbashir 2) indicated to us that siRNAs are structurally and mechanistically distinct from previously characterized antisense and ribozyme nucleic acid technologies. Based on this distinction, we did not expect the chemical modification patterns of ribozyme and antisense to be useful for determining successful chemical modification strategies for siRNA.

20. The publications in the art during about 2000–2001, including for example, the work of Zamore, Bass, Elbashir 1 and Elbashir 2, did in fact provide us general guidelines as to the design of active siRNA duplexes. These teachings can be summarized as follows: (a) double-stranded siRNAs are composed of 21 nt sense and 21 nt antisense siRNAs; (b) these siRNAs **must** be selected to form a 19 bp double helix; (c) the siRNA duplexes must contain 2 nt 3'-overhanging ends; (d) these 3'-overhanging nucleotide ends can be composed of two 2'-deoxythymidine nucleotides; (e) chemical modification of the internal positions within the siRNA duplex is not tolerated as they interfere the with RNAi activity of the siRNAs; (f) modifications other than 2'-deoxynucleotides at the 3'-end are not tolerated; (g) modification of the sense strand or the antisense strand fully with 2'-deoxynucleotides or 2'-O-methyl nucleotides abolish activity of the siRNAs to mediate RNAi, therefore demonstrating the need for the presence of ribonucleotides in the siRNAs for RNAi, and (h) chemical modification (e.g.,

2'-O-methylribose) of nucleotides in the internal region of the siRNA duplex abolished activity of siRNAs to mediate RNAi.

21. In 2001–2002, well before any of the published reports referenced above, we performed detailed, systematic analyses to determine the extent and pattern of chemical modifications that would be tolerated in siRNA duplexes; testing, for example, various modifications other than 2'-deoxy substitutions at the 3'-terminal positions of the siRNAs.

22. We first evaluated the serum stability of the siRNA constructs taught in Elbashir in comparison with duplexes having 2'-O-methyl, 2'-deoxy-2'-fluoro and/or 2'-deoxy ribose and other modifications at various positions. The constructs as taught by Elbashir had a stability half-life ($t_{1/2}$) of 15 seconds in human serum, compared to a $t_{1/2}$ of 32-40 days for the modified constructs we made. This work is described more fully in the instant application, which includes the experimental details and results of testing 2'-O-methyl, 2'-deoxy-2'-fluoro and/or 2'-deoxy ribose modified duplexes based on our systematic analysis of siRNA structure and function.

23. Surprisingly, and contrary to Elbashir 2, we discovered that extensive chemical modification of siRNA duplexes could be tolerated, even to the point where all ribonucleotides of the siRNA could be substituted without abolishing RNAi activity. Specifically, we found that modified duplexes with multiple 2'-O-methyl, 2'-deoxy-2'-fluoro and/or 2'-deoxy ribose (abasic) substitutions were highly potent mediators of RNA interference. We were the first ones to clearly demonstrate that, contrary to the teachings in the art, active siRNAs can be designed with chemical modifications at a number of positions, including at every position within the duplex.

24. Applying what we learned in these experiments to the design of modified duplexes targeting VEGFR1 resulted in active double stranded nucleic acid constructs with potent activity against VEGFR1 gene expression (see USSN 10/758,155, published as US-2005-0075304, USSN 10/831,620, published as US-2005-0148530, USSN 10/844,076, published as US-2005-0171039, USSN 10/944,611, published as US-2005-0233998, and USSN 10/962,898, published as US-2005-0222066).


25. Based on publications such as Elbashir 1 and Elbashir 2, it is my belief that one would not have been motivated to make siRNAs targeting the VEGFR1 RNA having one or

more 2'-O-methyl, 2'-deoxy-2'-fluoro and/or 2'-deoxy ribose modifications as described in US-2005-0075304, US-2005-0148530, US-2005-0171039, US-2005-0233998, and US-2005-0222066, because such publications indicated that such modifications would not be tolerated in an siRNA (see for example Elbashir 2).

26. In conclusion, a person working in the field of RNA interference at the time of filing the application for which I prepared this Declaration would not have been motivated to apply 2'-O-methyl, 2'-deoxy-2'-fluoro and/or 2'-deoxy ribose or other modifications to siRNAs merely because such modifications were used to stabilize antisense and ribozymes. In fact, the literature clearly demonstrated that the knowledge derived from antisense and ribozyme technologies for stabilizing oligonucleotides could not be readily applied to obtain active siRNAs (see, for example, Elbashir 2). It is my belief that those working in the general field of oligo- and polynucleotides for therapeutic use in the 2000–2002 timeframe would have believed, as we did, that the mechanism of RNA interference differed so significantly from both ribozymes and antisense oligonucleotides that the knowledge derived from those technologies likely could not be directly applied with any appreciable expectation of success. In fact, as discussed above, people in the art (see for example Elbashir 2) had tried the approaches used for antisense oligonucleotides and ribozymes to modify siRNAs (beyond the 3'-terminal nucleotides with deoxynucleotide) but failed to generate active siRNAs!

27. I hereby certify that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 3/21/2006

By: 
James McSwiggen